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Polymer-Mediated Ternary Supramolecular Interactions for Sensitive Detection of Peptides

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Abstract

A combination of donor-acceptor and electrostatic interactions in a three-component supramolecular system has been shown to form the basis for selective and sensitive detection of peptides. Different substituents in the polymer and the detection matrix were compared to demonstrate that favorable donor-acceptor interactions explain the observed signal enhancement. The ternary supramolecular interactions discovered in this work are enabled by the self-packing behavior of amphiphilic homopolymers and their ability to mediate interactions between the detection matrix and peptide that facilitate sensitive detection of peptides.

Graphical abstract

Executing a specific recognition event through ternary supramolecular interactions is quite common in Nature. Prominent examples involve adaptor proteins and enzymes that carry out coupling reactions precisely by first recognizing and binding to two different biomolecules and bringing them together.¹ Artificial molecular assemblies with such capabilities are relatively limited.^{2,3} In this manuscript, we show that amphiphilic homopolymers can act as a ‘molecular glue’ to effectively and specifically bring together two complementary molecules using disparate non-covalent interactions with each of the molecules. We show that these subtle supramolecular interactions manifest themselves in a macroscopic clustering phenomenon, which in turn has a significant effect on the highly sensitive detection of analyte peptides by matrix-assisted laser desorption/ionization – mass spectrometry (MALDI-MS) (Figure 1). Others have shown that matrix additives, analyte

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labelling or derivatization, and/or surface modification of the detection target can improve MALDI-MS signals.⁴ Our approach is different in that it relies on the inherent properties of the analyte and requires no such type of derivatizations or target surface modifications. Moreover, the same agent used for extraction enhances analyte detection. We report here on how both selectivity and sensitivity can be achieved through favourable ternary supramolecular interactions that can be tuned by variations in the structure of the interacting components. Formation of densely packed morphologies in the solid state through donor-acceptor supramolecular interactions has found applications in electronics materials,⁵ but the work here is the first study, to our knowledge, that tackles its role in mass spectrometric signal enhancement for sensitive molecular detection.

Recently, our group has developed amphiphilic homopolymers (Chart 1) that form kinetically-trapped reverse micelle assemblies in apolar solvents.⁶ We have shown previously that these assemblies are capable of selectively sequestering specific peptides based on their isoelectric point (pI) and solution pH.^{7,8} This electrostatically-driven interaction causes peptides, which are otherwise insoluble in apolar solvents, to be selectively moved to the apolar phase of a biphasic mixture (Figure S1). The versatility of this method has been illustrated by the highly sensitive MALDI-MS detection of peptides in complex mixtures such as protein digests.⁷ Furthermore, the general applicability of this approach in real samples has been demonstrated by the detection of pM concentrations of selected peptides in serum.⁸ These low detection limits are achieved not only from the selective enrichment that occurs upon extracting the peptides from an aqueous phase to an organic phase, but also from more than an order of magnitude enhancement observed in the MALDI ion signal after extraction. While it is understood that the amphiphilic homopolymer, exemplified by the polydialkoxystyrene (PDAS) shown in Chart 1, might play some role in this enhancement,^{7b} the underlying mechanism for such an enhancement is not understood, and is thus the subject of this study. Elucidating the molecular interactions responsible for this signal enhancement paves the way for improving detection sensitivities and can have tremendous impact on the analysis of complex peptide mixtures and on the detection of low-level peptide and protein biomarkers.

The first clue about the molecular basis for this signal enhancement came from the observation that a polyacrylamide, PAm in Chart 1, does not provide any signal enhancement (Figure 2). This polymer has been shown to form reverse micelle assemblies,⁹ similar to those formed by PDAS. We find that this tendency to form an assembly translates to selective sequestration of peptides based on pI in a manner similar to that of PDAS (characterization of the polymers in terms of reverse micelle formation and selectivity of extraction are shown in Figures S6–S12). The key difference is that there is MS signal enhancement only in the case of PDAS (Figure 2). Interestingly, there is an associated macroscopic cluster formation in the ternary mixture formed between PDAS, the analyte peptide, and MALDI matrix, α -cyano-4-hydroxycinnamic acid (CHCA), that does not occur in the presence of PAm (Figure 2). This led us to hypothesize that the electron-rich aromatic ring of the PDAS polymer is engaged in a donor-acceptor interaction with the electron-poor aromatic ring of CHCA, while the polymer's carboxylate unit is involved in interacting with the electrostatically complementary peptide. Although these interactions can presumably exist in the presence of just the monomer bearing the aromatic ring and the carboxylate

moiety, results from experiments where the monomer was added to the peptide-matrix mixture in place of its corresponding polymer reveal no significant enhancement in signal, suggesting that the multivalent structure of the polymer is needed for signal enhancement (Figure S2). Likewise, using the polymer as merely a matrix additive without doing any extraction does not lead to signal enhancement, as we demonstrated previously.^{7a} Thus, upon extraction the polymer structure acts as the effective mediator between the matrix molecule and the peptide.

To test this hypothesis, we first varied the matrix from CHCA to α -cyanocinnamic acid (CCA) and α -cyano-4-chlorocinnamic acid (CCICA). By replacing the $-\text{OH}$ group with $-\text{H}$ or $-\text{Cl}$, the electron-withdrawing ability of the substituent is varied in the order $-\text{OH} < -\text{H} < -\text{Cl}$. Upon extraction and detection of the positively charged, TAMRA-labeled bradykinin peptide (RPPGFSPFR), we find that the MS signal is enhanced by 20% with CCA as the matrix, and more than 100% with CCICA, compared to that of CHCA as the matrix (Figure 3). Moreover, corresponding fluorescence images reveal that clustering of the extracted peptide is the densest with the CCICA matrix (Figure 3). This observation is consistent with the hypothesis that the electron-rich PDAS polymer would interact better with more electron-poor matrices, which in turn causes more clustering and affords greater signal enhancement.

To further test our hypothesis, we investigated how varying the electron density on the aromatic ring of the polymer affects MALDI signal enhancement and hotspot formation, while keeping the MALDI matrix constant. In this case, we introduced aromatic rings with electron-donating or electron-withdrawing substituents in an amphiphilic homopolymer, shown as PTLAm in Figure 4 (synthetic details in SI). These polymers also form reverse micelles and selectively extract positively-charged peptides (Figures S6–S7). Consistent with our hypothesis, using the PTLAm polymer with the electron-donating methoxy substituent (PTLAm-OMe) resulted in a two-fold enhancement in MALDI signal relative to the unextracted peptide (Figure 4). Interestingly, the signal was significantly suppressed, when PTLAm-NO₂, with its electron-withdrawing nitro group, was used. The unsubstituted PTLAm polymer (PTLAm-H) did not show a significant difference in MALDI signal relative to the unextracted one. Furthermore, clustering of peptide and matrix into hotspots is apparent in the presence of the PTLAm-OMe, but not for either PTLAm-H or PTLAm-NO₂, suggesting that a polymer with an electron-rich aromatic ring is needed for hotspot formation and signal enhancement.

While the PTLAm polymers provided access to amphiphilic polymers with aromatic units, we were interested in a scaffold that could provide the opportunity to conveniently introduce subtle changes in the aromatic unit, such as a wide range of electron-donating or -withdrawing substituents on the ring, while keeping the overall structure consistent with the PAm structure shown in Chart 1. Accordingly, we designed and synthesized (see SI for details) a new set of amphiphilic homopolymers with a polyacrylamide (PAm) scaffold (Chart 2). These polymers provide the opportunity to further test our hypothesis that a ternary supramolecular interaction between the polymer, matrix and the peptide is essential for enhanced detection capabilities. Six polymers with different electron-withdrawing and electron-donating substituents were synthesized (Chart 2). All these polymers self-assemble

into nanometer-sized reverse micelles (Figures S8–S10) and selectively extract oppositely charged peptides (Figures S11–S12), just like the PDAS and PAm polymers.

The degree of matrix-peptide clustering and MALDI signal enhancement after extraction was monitored by fluorescence microscopy and MS, respectively. Through mere visual inspection (Figure 5), it is evident that a higher degree of clustering is present in samples extracted by polymers with electron-donating groups (PAH, PAm-Me, PAm-OMe, PAm-di-OMe) compared with those extracted by polymers with electron-withdrawing groups (PAm-NO₂ and PAm-CN). At first glance, we would expect PAm-H to behave similar to the control (unextracted) sample, because of the –H substituent. Note, however, that the base aromatic ring is based on an alkoxyarene, making PAm-H a relatively electron-rich aromatic ring. We quantitatively assessed the degree of hotspot formation by measuring the fluorescence intensity per area using the software ImageJ (Figure S14) and correlated this with the MALDI-MS signal obtained for samples extracted by each polymer. Figure 5 reveals that an approximately 3-fold enhancement in signal is obtained when polymers with electron-donating substituents are used for extraction, whereas no appreciable enhancement in signal was observed when extracted by polymers with electron-withdrawing groups. Correspondingly, higher degrees of hotspot formation were calculated for samples extracted using PAm-H, PAm-Me, PAm-OMe and PAm-di-OMe compared to PAm-NO₂ and PAm-CN. This correlation shows that the clustering phenomenon indeed translates to enhanced signals in MALDI-MS. Because good co-crystallization between the peptide and the MALDI matrix is also crucial to obtaining strong MS signals, we measured the degree of co-localization between the TAMRA-labeled peptide and the MALDI matrix for each of the fluorescence micrographs (Figure S15) and found an index of correlation of >0.800 for all images. This high correlation suggests very good co-localization between the peptide and the matrix regardless of the polymer used. Thus, the signal enhancement is not simply due to better analyte/matrix co-crystallization. Instead, the formation of “hotspots” through the use of polymers with favorable donor-acceptor interaction with the MALDI matrix is key to obtaining the signal enhancement.

Conclusions

In summary, we have investigated the molecular basis for the observed signal enhancement in peptide detection by MALDI-MS, after extraction using reverse micelles of amphiphilic polymers. Through systematic variations in the electronic character of the aromatic ring in the amphiphilic polymer and matrix, we find that favourable donor-acceptor interactions are necessary to form a ternary assembly of the polymer, peptide, and matrix, which is essential for producing peptide-rich zones that maximize ion signal in the mass spectrum. An important point to recognize from all the data presented here is that although the electrostatic interaction between the polymer and the peptide is required to selectively extract peptides from the aqueous phase into the organic phase, these interactions alone are not sufficient to cause hotspot formation and signal enhancement. All these polymers bear the carboxylate moiety that can conceivably cause the peptides to coalesce into hotspots, but evidently, a favourable aromatic donor-acceptor interaction between the polymer and the matrix is necessary for hotspots to form and signal enhancement to manifest. Thus, the ternary supramolecular interaction formed between the polymer and the peptide through

electrostatics, and between the polymer and the matrix through aromatic donor-acceptor interactions, forms the basis for the selective enrichment and sensitive detection of peptides. Overall, these results reveal that amphiphilic polymers can self-pack in such a way that they mediate favourable interactions between peptides and matrix. Such interactions are reminiscent of proteins that upon folding, position other biomolecules in just the right way to perform chemistry that is impossible without the ternary interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation of amphiphilic polymers self-assembling into reverse micelles for the selective extraction of peptides based on pI. The ternary interactions involving the amphiphilic homopolymer assembly with the analyte peptide through ionic complementarity and with the detection matrix through aromatic donor-acceptor interactions are shown. Stronger ternary molecular interactions result in significant enhancement in the peptide detection signal.

Figure 2.

MALDI mass spectra for unextracted 1 μ M* bradykinin (A), and 10 nM* bradykinin (RPPGFSPFR, m/z 1060.6) extracted using the PDAS polymer (B), and the PAm polymer (C). Corresponding fluorescence images showing the degree of clustering and co-crystallization of TMR-bradykinin peptide (red) and CHCA matrix (green). Scale bar = 100 μ m. Fluorescence images of individual and merged channels to illustrate co-localization of the three components are shown in Figure S3. *100-fold higher concentration in the unextracted sample (1 μ M) relative to extracted sample (10 nM) to account for the 100-fold enrichment during extraction.

Figure 3. Chemical structures of the different MALDI matrices in order of increasing electron deficiency on the ring, along with the corresponding MALDI mass spectra and fluorescence images obtained for 100 nM bradykinin extracted using the PDAS polymer (Chart 1) and analysed using these MALDI matrices. Peaks with asterisks are potassium adducts of the peptides, $[M+K]^+$. Fluorescence images of individual and merged channels of peptide with matrix to illustrate co-localization are shown in Figure S4.

Figure 4. MALDI signal intensity of 10 nM bradykinin extracted by polythiolactone amide (PTLAm)-based carboxylate polymers with different R groups compared with 1 μ M of unextracted bradykinin (100-fold higher concentration to account for 100-fold enrichment during extraction). Fluorescence images show the degree of clustering of peptide (red) and CHCA matrix (green) after extraction using each PTLAm polymer. Fluorescence images of individual and merged channels to illustrate co-localization of the three components are shown in Figure S5.

Figure 5.

Fluorescence images (top panels; scale bar=100 μm) showing the degree of clustering of peptide (red) and CHCA matrix (green) upon extraction using the PAm-based polymer indicated, and correlation of the degree of clustering with the MALDI-MS signal (bottom graph). Each blue bar represents the average ± SEM of 90 spectra obtained from 3 replicate extractions with 3 spots each. Fluorescence images of individual and merged channels to illustrate co-localization of the three components are shown in Figure S13.

Chart 1.
Chemical structures of poly(dialkoxystyrene)-based and polyacrylamide-based amphiphilic polymers.

Chart 2.

Chemical structures and labels of the polyacrylamide-based amphiphilic homopolymers with the carboxylate hydrophilic moiety and variable hydrophobic units containing electron-donating or electron-withdrawing functionalities.